Obesity and Bariatric Surgery Drive Epigenetic Variation of Spermatozoa in Humans

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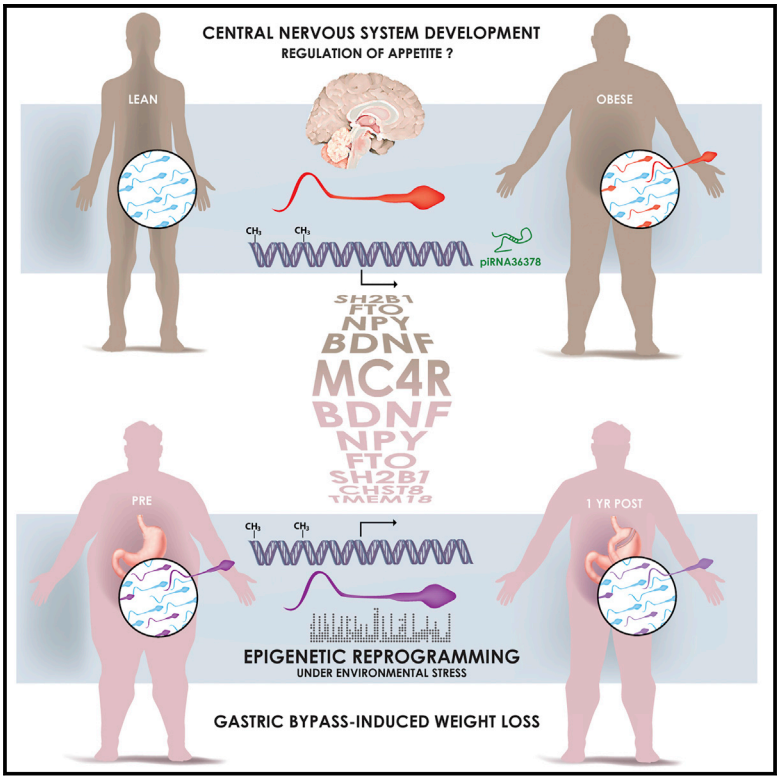
Graphical Abstract



Highlights

- Distinct sncRNA expression and DNA methylation profiles in sperm from obese humans
- Differentially methylated genes are related to brain function
- The spermatozoal epigenome is dynamically remodeled after bariatric surgery
- Differential methylation clusters with known SNPs of obesity

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In Brief

Donkin et al. show that spermatozoa from obese men carry a distinct epigenetic signature compared to lean men, in particular at genes controlling brain development and function. The sperm methylome is dynamically remodeled after gastric-bypass-induced weight loss, notably at gene regions implicated in the central control of appetite.

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Short Article

Obesity and Bariatric Surgery Drive Epigenetic Variation of Spermatozoa in Humans

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SUMMARY

Obesity is a heritable disorder, with children of obese fathers at higher risk of developing obesity. Environmental factors epigenetically influence somatic tissues, but the contribution of these factors to the establishment of epigenetic patterns in human gametes is unknown. Here, we hypothesized that weight loss remodels the epigenetic signature of spermatozoa in human obesity. Comprehensive profiling of the epigenome of sperm from lean and obese men showed similar histone positioning, but small non-coding RNA expression and DNA methylation patterns were markedly different. In a separate cohort of morbidly obese men, surgery-induced weight loss was associated with a dramatic remodeling of sperm DNA methylation, notably at genetic locations implicated in the central control of appetite. Our data provide evidence that the epigenome of human spermatozoa dynamically changes under environmental pressure and offers insight into how obesity may propagate metabolic dysfunction to the next generation.

INTRODUCTION

Obesity is a metabolic disorder resulting from behavioral and heritable causes. Children of obese fathers are at higher risk of developing metabolic disease later in life, independent of the body weight of their mother (Lake et al., 1997), supporting the notion that paternal factors contribute to the inheritance of obesity and obesity-related traits. While the socioeconomic status of the father could be involved, studies in rodents and epidemiological data highlight that paternal nutritional status can directly affect the health of the offspring (Carone et al., 2010; Fullston et al., 2013; Kaati et al., 2002, 2007; Ng et al., 2010; Pembrey et al., 2006; Wei et al., 2014), suggesting that an environmentally acquired phenomenon of epigenetic inheritance is passed on by the gametes. Exercise and nutritional status induce acute changes in DNA methylation patterns in human skeletal muscle and adipose tissue (Barres et al., 2013; Barrès et al., 2012; Brøns et al., 2009; Jacobsen et al., 2014; Multhaup et al., 2015; Rönn et al., 2013), demonstrating that environmental factors remodel the epigenome of somatic tissues. Whether obesogenic factors like caloric excess and sedentarity can change the epigenome of human gametes is unknown.

Here, we hypothesized that weight loss remodels the epigenetic signature of spermatozoa in human obesity. We present the first epigenetic mapping of spermatozoa in obese men and reveal a distinct epigenome that characterizes human obesity. We show that sperm DNA methylation profiles are changed after bariatric surgery, indicating that the epigenetic landscape of human sperm is dynamic and vulnerable to environmental changes.

RESULTS

To analyze the epigenetic patterns of spermatozoa in obesity, we collected single ejaculates from lean, normal-glucose tolerant (median body mass index, BMI, of 22.9, referred to as "Lean") and obese, glucose intolerant men (median BMI of 31.8, referred to as "Obese"). Clinical characteristics are detailed in Table S1 and graphically summarized in Figure 1A. Micrococcal nuclease (MNase)-sequencing (seq), small non-coding RNA (sncRNA)-seq, and reduced representation bisulfite Seq (RRBS) were performed to characterize histone positioning, sncRNA expression, and DNA methylation, respectively, on pure, motile spermatozoal fractions.

Spermatozoal Histone Positioning Is Not Altered between Lean and Obese Men

In spermatozoa, the main part of genomic DNA is wrapped around protamines, allowing for denser packaging of DNA compared with histones. Previous work suggests that the

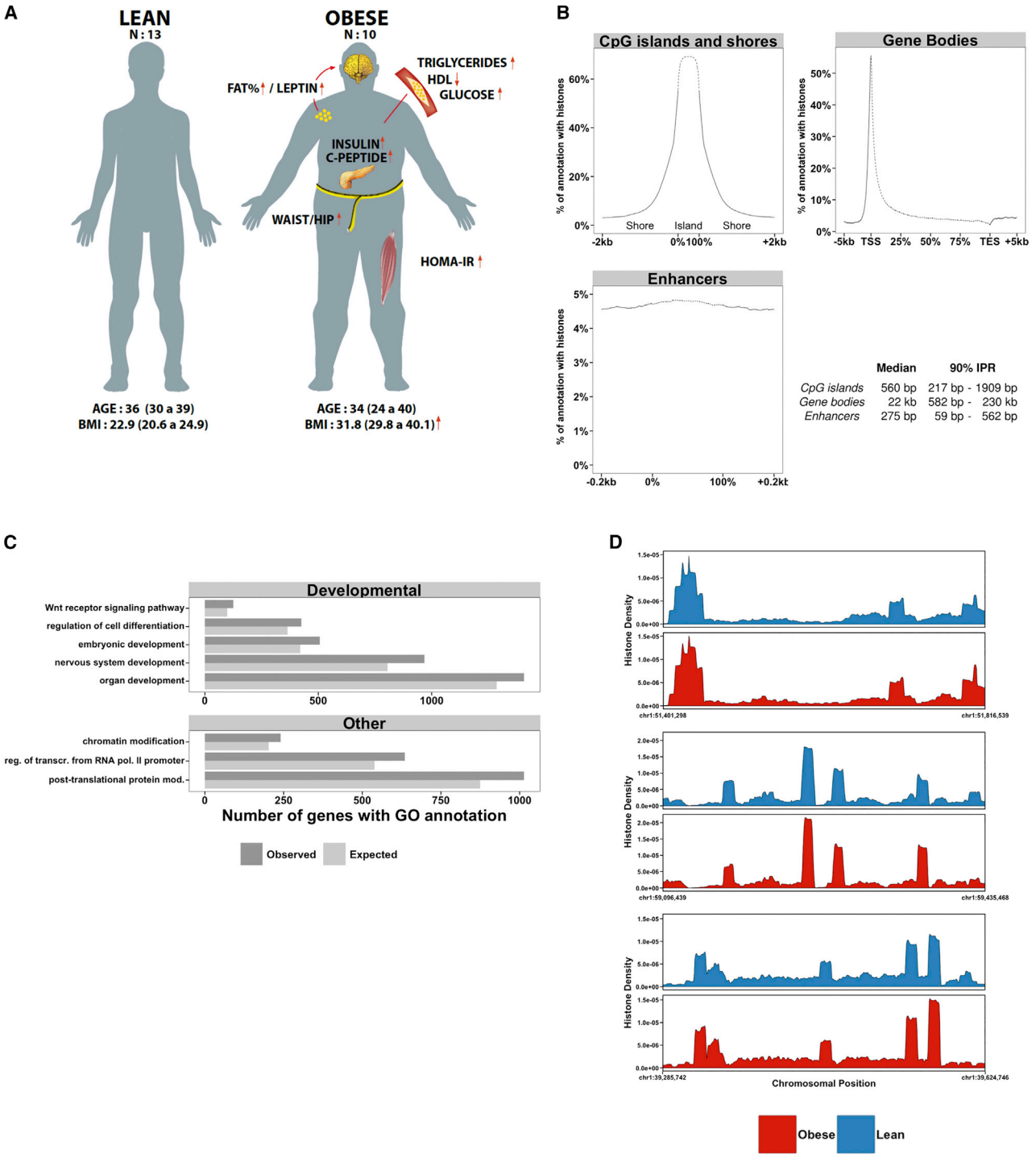

Figure 1. Spermatozoal Histone Positioning Is Unchanged in Human Obesity

The genomic location of histone retention by MNase-seq in Lean and Obese men is shown.

(A) Clinical characteristics of the Lean and Obese men (arrows, Obese versus Lean; homeostatic model assessment of insulin resistance, HOMA-IR; high-density lipoprotein cholesterol, HDL; glucose 2 hr after 75 g of glucose ingestion, Glucose). See Table S1 for all clinical characteristics.

(B) Histone retention at CpG islands and shores, gene bodies, and enhancer regions, across all samples. Due to varying region lengths, regions are plotted as percentages (stippled range) and x axes are scaled to the median lengths of the respective regions (transcription start site, TSS; transcription end site, TES; inter-percentile Range, IPR).

(C) Selected GO terms enriched in genes carrying histones, across all samples. See also Table S2.

(D) Examples of histone retention levels in Lean and Obese men across chromosome 1. The signal was generated using macs2 bdgcmp with the "-m ppois" parameter set.

epigenomic packaging of genes around either nucleosomes or protamines control early development of the embryo (Arpanahi et al., 2009; Brykczynska et al., 2010; Hammoud et al., 2009). Our analysis of the genomic regions with retained histones in Lean and Obese men showed histone retention at approximately 2% of the genome. We found histone retention was enriched in CpG islands and around transcription start sites (TSS) (Figure 1B). Approximately 5% of enhancer regions reported in somatic cells (Andersson et al., 2014) retained histones in mature sperm (Figure 1B). Gene ontology analysis using the bioinformatics tool DAVID (Eden et al., 2009; Huang et al., 2009a, 2009b) revealed that genes carrying histones are enriched for the Gene Ontology (GO) terms "Metabolic Process" and "Developmental Process" and related subgroups (Figure 1C; Table S2). Specific enrichment of histone retention around TSS and at genes regulating developmental processes is consistent with previous reports in human semen (Arpanahi et al., 2009; Brykczynska et al., 2010; Hammoud et al., 2009). But, importantly, histone positioning was unaltered in spermatozoa between Lean and Obese men (Figure 1D).

Spermatozoa from Obese Men Have Altered Expression of sncRNAs

sncRNAs constitute a subtype of RNAs that are implicated in epigenetic inheritance. Piwi-interacting RNAs (piRNAs) are mainly expressed in the germline and play a fundamental role in genomic stability by repressing repetitive elements (Siomi et al., 2011), as well as in the regulation of coding gene expression (Esposito et al., 2011). A role for piRNAs in epigenetic inheritance has been demonstrated in *Drosophila* (Brennecke et al., 2008) and *C. elegans* (Ashe et al., 2012). Analysis of the spermatozoal sncRNA content from Lean and Obese men revealed that the most abundant sncRNA subtypes were piRNAs and tRNA fragments (tRFs). Consistent with previous observations in healthy men (Krawetz et al., 2011), microRNAs (miRNAs) represented only a small fraction of the total sncRNA content (Figures 2A and 2B; Table S3). Although the distribution of sncRNA subtypes was unchanged (p values > 0.05, Kolmogorov-Smirnov (KS) test; Figure 2C), the expression level of specific miRNAs, piRNAs, tRFs, and small nuclear RNA (snRNA) fragments was altered in the spermatozoa from Obese men (Figure 2D; Table S3). While differential expression of miRNAs, tRFs, and snRNA fragments did not survive corrections for multiple testing, we identified 37 piRNAs with a false discovery rate (FDR) below 0.1. Target prediction of the piRNAs differentially expressed between Lean and Obese men retrieved genes with best enrichment scores for the GO terms "Chromosome" and "Chromatin" and the genetic annotation term "Chemdependancy". Specifically, the cocaine and amphetamine regulated transcript (*CART*), a regulator of food intake involved in obesity (Asnicar et al., 2001; Hunter et al., 2004) (Table S4), was differentially expressed in Obese men. We speculate that this altered piRNA expression coordinately modulates the expression of genes involved in behavior and food intake and could participate in predisposing the offspring to obesity.

Genes Regulating the Development and Function of the CNS Are Differentially Methylated in Spermatozoa from Obese Men

Given that DNA methylation status at specific locations in the genome of the developing embryo is inherited through the paternal line (Rakyan et al., 2003), and that zygotic demethylation at the paternal genome after fertilization is associated with transcriptional activation in the embryo (Peat et al., 2014), altered DNA methylation changes in sperm from obese men would likely alter the development of the embryo and the phenotype of the offspring. This could explain why paternal obesity is associated with altered DNA methylation at imprinted genes in the cord blood cells of the offspring (Soubry et al., 2013). Thus, we next characterized DNA methylation in motile spermatozoa collected from the same ejaculates as described above (Table S1). We identified 9,081 unique genes differentially methylated between Lean and Obese men (Table S5). In all regions of the genome, we found either close to no methylation or very high methylation levels. Consistent with a previous study describing hypomethylation at open chromatin (Hammoud et al., 2009), methylated CpGs were almost exclusively found in protamine-associated DNA, irrespectively of obesity (Figure 3A). The number ($p < 0.0001$, chi-square test) and magnitude ($p < 0.0001$, KS test; Figure 3B) of CpG methylation changes found between Lean and Obese men were also higher in protamine-bound regions, compared to histone-retained regions, suggesting polymorphism in CpG methylation occurs at specific locations in the spermatozoal genome. A GO analysis of the genes with differentially methylated CpGs revealed enrichment for the term "nervous system development" (Figure 3C; Table S6). While the representation of the enrichment is graphically modest (Figure 3C), the statistical significance is substantial. Such discrepancy between graphical representation and level of statistical significance is consistent with another epigenomic study (Lin et al., 2009) and is likely due to the large amount of genes investigated. An analysis for previously identified genetic variants related to disease from the Genetic Association Database (Becker et al., 2004) returned the term "Psychiatry" ($p = 0.038$), although this did not survive correction for multiple testing ($p = 0.052$). In the gene list containing 274 genes, we identified several master regulators of appetite control including melanocortin-4 receptor (*MC4R*), brain-derived neurotrophic factor (*BDNF*), neuropeptide Y (*NPY*), cannabinoid receptor type 1 (*CR1*), *CART*, and genes related to obesity and metabolism including fat mass and obesity associated (*FTO*), carbohydrate sulfotransferase 8 (*CHST8*), and SH2 binding domain-containing protein 1 (*SH2B1*) (Figure 3D) (Branson et al., 2003; Pi-Sunyer et al., 2006; Urabe et al., 2013; Willer et al., 2009). These results suggest a specific remodeling of epigenetic marks for genes controlling the function of the CNS and metabolism. This is consistent with a recent study showing that genes involved in neurological disorders and metabolism escape demethylation in the human primordial germ cell, suggesting that certain genomic regions, specifically, genes at the intercross between the CNS and the regulation of metabolic function, are hotspots for epigenetic variability in gametes (Tang et al., 2015).

Genetic polymorphisms can influence methylation at proximal cytosine residues (Hitchins et al., 2011). To investigate if epigenetic variation between Lean and Obese could be caused by genetic polymorphisms, we genotyped 23 known SNPs associated with obesity (Thorleifsson et al., 2009) in Lean and Obese subjects. None of the investigated SNPs were positioned within a distance of 1,000 bp of a differentially methylated cytosine. Additional analysis of SNPs located within 100,000 bp from the *NPY*, *CART*, *CR1*,

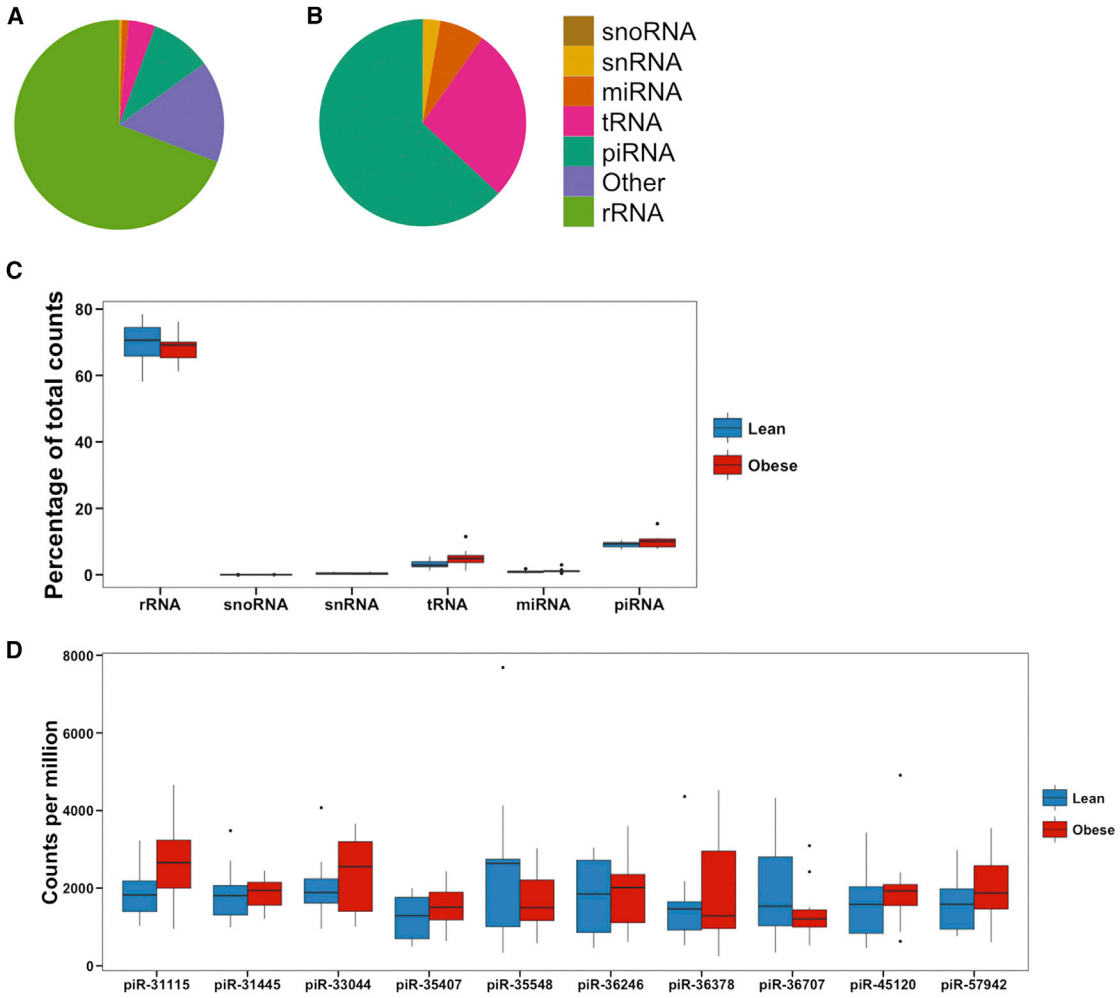

Figure 2. Altered piRNA Content in Spermatozoa from Obese Men

(A and B) Average abundance of sncRNA subtypes across Lean and Obese samples, from sncRNA-seq data (A) and after rRNA and other RNA removal (B) (exact percentages can be found in Table S3).

(C) Median percentages and 25th and 75th percentiles of sncRNA subtypes in Lean and Obese men. The percentages in (A)–(C) are normalized to total amount of sequenced preprocessed reads per sample.

(D) Median expression levels and 25th and 75th percentiles of selected differentially expressed piRNAs between Lean and Obese men. The counts per million (CPM) are normalized to total number of piRNA reads per sample. See Table S4 for a list of predicted piRNA targets.

and *MC4R* genes using a principal component analysis showed no relationship between the genotype and epigenetic variability in Lean and Obese (Figure S1), excluding a contribution of genetic variants to the observed differential methylation.

In addition to an increased risk for obesity (Lake et al., 1997), children of obese men are at higher risk of developing autism spectrum disorders (ASD) (Murphy, 2014; Surén et al., 2014). We compared our results to a recent study reporting DNA methylation changes in sperm from fathers having infant siblings diagnosed with ASD (Feinberg et al., 2015). Strikingly, DNA methylation changes in the ASD enriched-risk cohorts are also enriched for genes regulating the development of the CNS (Feinberg et al., 2015). We found a substantial overlap of differentially methylated genes between the Lean/Obese cohort and the ASD enriched-risk cohort (79%, $p = 3.5 \times 10^{-12}$). These observations reinforce the notion that spermatozoa in obese men are epigenetically reprogrammed for genes controlling the CNS, which may participate in an altered social and feeding behavior in children of obese men.

DNA Methylation in Spermatozoa Is Remodeled after Bariatric Surgery-Induced Weight Loss

To directly investigate the potential influence of weight loss on the epigenome of human sperm, we studied the spermatozoal DNA methylation profiles in obese men before and after bariatric Roux-en-Y gastric bypass (GBP) surgery. The Roux-en-Y GBP is a potent intervention to reduce weight and resolve insulin resistance and type 2 diabetes (Dirksen et al., 2012). Single ejaculates were collected from morbidly obese men undergoing Roux-en-Y GBP surgery at three time points: approximately 1 week before (median BMI of 42.6, referred to as "pre-GBP"), 1 week after ("GBP-1w", median BMI of 40.1, $p < 0.05$ compared to pre-GBP)

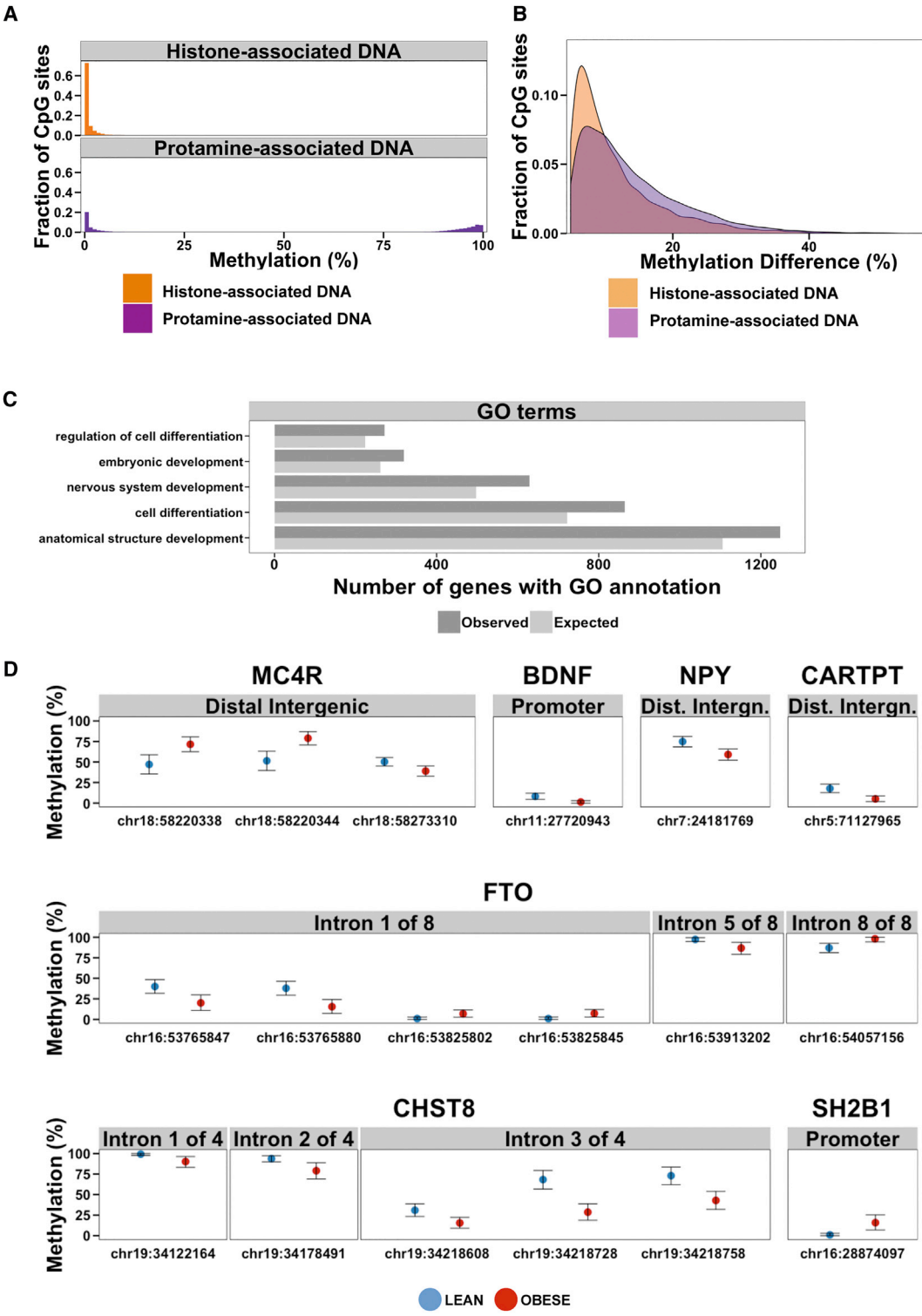

Figure 3. Sperm DNA Methylation at Specific CpG Sites Is Changed in Obesity

CpG methylation patterns by RRBS in Lean and Obese men. See Table S5 for the full list of genes.

(A) Protamine-bound regions show a bimodal methylation distribution contrasted by the unmethylated profile of histone-retained regions, irrespective of the group.

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and 1 year after the surgery ("GBP-1y", median BMI of 33.9, $p < 0.05$ compared to pre-GBP) when weight loss had stabilized. Each ejaculate was purified and analyzed individually for DNA methylation. Clinical characteristics are detailed in Table S7 and Figure 4A. Using paired analyses, we found the methylation status of 1,509 unique genes were changed 1 week after the surgery (GBP-1w versus pre-GBP) (Figure 4B; Table S8) and 3,910 unique genes were differentially methylated in sperm from GBP-1y compared to pre-GBP (Figure 4B; Table S9), suggesting that weight loss induces an alteration of the sperm epigenome. Our observation that sperm DNA methylation was remodeled rapidly after GBP surgery suggests that epigenetic changes can occur during the last stages of sperm maturation. This is supported by the observation that DNA methyltransferases are present in mature sperm (Marques et al., 2011). Of the 1,509 genes altered at GBP-1w, 1,004 genes and 1,116 CpG positions were also differentially methylated in GBP-1y, after stable weight loss (Figure 4B), suggesting epigenetic variation occurs at specific sites in the sperm genome. Absolute methylation patterns of Lean, Obese, and at the three different time points in men undergoing GBP show a correlation of 0.98–0.99, indicating low inter-individual variation. However, these patterns can change with environmental factors; we found that 2,681 of the genes with changed DNA methylation in GBP-1y compared to pre-GBP were also altered between Lean and Obese men (Figure 4C; Table S10). Even though this overlap was not significant, it nevertheless indicates certain "hotspots" for methylation changes in the genome associated with changes in nutritional intake. Remarkably, numerous differentially methylated genes common between Lean versus Obese and pre-GBP versus GBP-1y cohorts were previously reported as carriers of genetic polymorphism associated with severe onset of obesity, such as transmembrane protein 18 (*TMEM18*), *CHST8*, *SH2B* adaptor protein 1, *BDNF*, *FTO*, and *MC4R* (Thorleifsson et al., 2009; Willer et al., 2009) (Figure 4D). None of the study participants carried a SNP associated with obesity, indicating GBP-induced weight loss modulates the epigenetic landscape of spermatozoa and alters specific genomic regions, notably of master genes that have previously been identified in genome-wide association studies of obesity.

To investigate whether the differentially methylated cytosines identified after GBP were also changed in somatic tissues, we compared methylation of 60 CpG sites in spermatozoa with blood and subcutaneous fat tissue collected from subject's pre-GBP and GBP-1y. Within all cytosines investigated, we observed distinct methylation patterns between the tissues (Figure S2A). Principal component analysis showed that methylation levels between time points were tissue-specific (Figures S2B and S2C). Collectively, these results provide evidence to suggest that environmental factors associated with GBP induce a remodeling of epigenetic marks that is unique to spermatozoa.

Our data provide evidence that human gametic epigenetic variation can be related to nutritional status. In an attempt to identify the factors contributing to epigenetic changes in sperm cells, we performed a principal component analysis of DNA methylation changes against the clinical parameters of the study participants. Linear regression of the first six principal components did not return any association between DNA methylation and the clinical parameters listed in Table S1. Future investigations to identify the particular changes in the testicular milieu in obesity or after weight loss may reveal the specific factors that contribute to the dynamic epigenetic changes observed in sperm cells.

In conclusion, we have identified altered epigenetic profiles in spermatozoa from men with moderate obesity and found that the sperm methylome is altered after GBP surgery in morbidly obese humans. The extent to which these gametic epigenetic changes in obese men influence the metabolic profile of their offspring is unknown. Animal studies have provided contradictory results regarding any direct transmission of the spermatozoal epigenome to metabolic tissues of the offspring (Carone et al., 2010; Radford et al., 2014; Wei et al., 2014). Thus, future studies contrasting the epigenome of gametes with that of somatic tissues obtained from the offspring are warranted to isolate the specific epigenetic marks that reprogram the metabolic phenotype of humans. A wide variety of environmental stressors are likely to influence the epigenome of spermatozoa. Future endeavors to characterize the epigenetic profile of human spermatozoa upon other environmental exposures, including exercise or smoking, may reveal distinct signatures that may influence the metabolic health of subsequent generations in a positive or deleterious manner. A greater understanding of the mechanism controlling epigenetic inheritance may raise societal awareness of preconceptional behavior.

EXPERIMENTAL PROCEDURES

Study Cohorts

Semen samples were collected from 23 Caucasian age-matched male volunteers in their reproductive age (20–40 years); 13 were lean (BMI 20–25) and ten obese (BMI > 29.7). In addition, single ejaculates were collected from six Caucasian obese men undergoing Roux-en-Y GBP surgery at three time points: approximately 1 week before, 1 week after, and 1 year after the surgery when weight loss had stabilized. Some of the blood samples from the study participants from these two cohorts were used in a previous study (Simar et al., 2014). Study cohorts are further detailed in Supplemental Information.

Isolation of Motile Spermatozoa

A "swim-up" procedure was performed to exclude somatic cells and to isolate motile spermatozoa. The upper fractions were pooled per ejaculate and the spermatozoa counted by microscopy. Presence of somatic cells was inspected. Isolation of motile spermatozoa is further detailed in Supplemental Information.

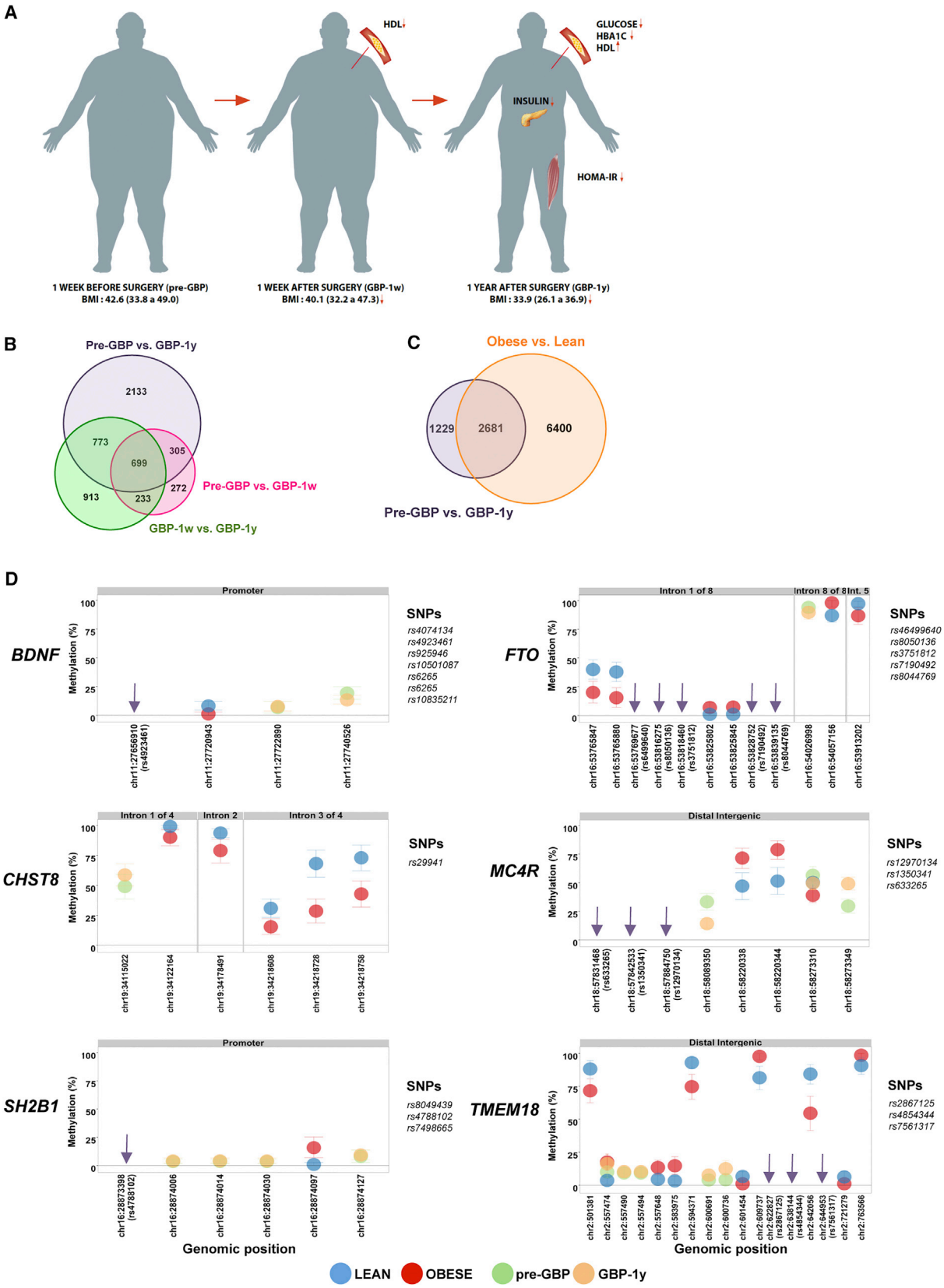
Collection of Blood and Subcutaneous Fat

Venous blood was collected in EDTA tubes after venous puncture of the antecubital fossa. Subcutaneous fat biopsies from the lower periumbilical area were collected with a Bergström needle with suction under local anesthesia

(B) In comparison to histone-retained DNA, protamine-bound DNA shows higher methylation changes between Obese and Lean men. The methylation changes are in absolute values.

(C) Selected GO terms enriched in genes with differentially methylated CpGs between Obese and Lean men. See also Table S6.

(D) Examples of differentially methylated genes involved in the regulation of appetite control and metabolism. The circles represent the median values of methylation levels, and the whiskers represent the 95% Bayesian highest density intervals.



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and immediately rinsed in PBS and snap frozen in liquid nitrogen until further analysis.

Histone Positioning, sncRNA Expression, and DNA CpG Methylation Analyses

All epigenetic analyses were performed on single ejaculates. For histone positioning analysis, we adapted a protocol previously described (Hammoud et al., 2009). Sample and library preparation are further detailed in Supplemental Information. SncRNA-Seq libraries were prepared using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs), according to the manufacturer's instructions. For DNA CpG methylation analysis, we prepared RRBS libraries constructed as previously described (Boyle et al., 2012).

Bisulfite-Seq

Extracted genomic DNA from semen cells, blood cells, and subcutaneous fat was bisulfite treated using the EZ DNA Methylation Lightening Kit (Zymo Research) according to the manufacturer's instructions. The PCR amplified bisulfite converted products were ligated to TruSeq (Illumina) sequencing adaptors before sequencing on a MiSeq Illumina Instrument to allow for comparative analysis of CpG methylation at selected targets in gametic and somatic cells from the same individual before and after bariatric surgery.

Genotyping

The presence of SNPs in genomic DNA from Lean and Obese individuals was analyzed by the Infinium CoreExome-24 BeadChip (Illumina) using DNA extracted from blood cells with DNeasy Blood and Tissue Kit (QIAGEN) as according to the manufacturer's recommendations.

Analysis of Seq Data

Analysis was performed in R using Bioconductor packages. For histone retention data, preprocessed reads of at least 15 nt were aligned to hg19 with Bowtie2 (Langmead et al., 2009). Retention peaks were called by MACS2 peakcall (Langmead et al., 2009), excluding duplicate reads. Differential histone retention was identified with DiffBind (Ross-Innes et al., 2012). For sncRNA data, preprocessed reads of at least 15 nt were aligned to hg19 with Bowtie (Langmead et al., 2009). Differential RNA expression was calculated by edgeR (Robinson et al., 2010). piRNA targets were predicted by searching their genomic locations to find overlapping or nearest genes and annotated with ChIPpeakAnno (Zhu et al., 2010).

For RRBS data, preprocessed reads of at least 15 nt were aligned to hg19 with Bismark (Krueger and Andrews, 2011). Differential methylation was analyzed with methylKit (Akalin et al., 2012), adapted to model the paired nature of the GBP cohort by extending the internal model with a term corresponding to the individual subject. All regions were annotated with the bioconductor package ChIPseeker (Yu et al., 2015) R package version 1.2.5, http://www.bioconductor.org/packages/release/bioc/html/ChIPseeker.html). Only CpGs with more than 5% methylation change and with a q-value below 0.1 were included for further analysis.

GO analysis, through DAVID (Eden et al., 2009; Huang et al., 2009a, 2009b) and by using all biological process terms (GOTERM_BP_ALL), was used to analyze the biological functions of genes with methylation changes at any genomic location, genes carrying histones at their promoter (5,000 bp up- and 1,000 bp downstream from the TSS) and within gene bodies, or genes predicted to be targeted by differentially expressed piRNAs. For the DNA methylation data sets, background was set to all measured genomic regions. An analysis for previously identified genetic variants related to disease from the Genetic Association Database was from the GENETIC_ASSOCIATION_DB_DISEASE_CLASS cluster. Bioinformatic analyses are further detailed in Supplemental Information.

ACCESSION NUMBERS

Seq data have been archived in a publicly accessible database at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) with accession number GEO: GSE74426.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and ten tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2015.11.004.

AUTHOR CONTRIBUTIONS

I.D., S.V., J.R.Z., and R.B. designed the study. I.D., S.V., M.M., L.N., B.M., E.V.R.A, N.J., and V.B.K. gathered the data. I.D., S.V., L.R.I., K.Q., M.M, C.T.W., T.H., and R.B. analyzed the data. I.D., S.V., J.R.Z., and R.B. wrote the paper. All authors vouch for the data and the analysis and agreed to publish the paper.

CONFLICTS OF INTEREST

The authors have no competing financial interests to declare.

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Figure 4. Altered Spermatozoal DNA Methylation after GBP Surgery-Induced Weight Loss

CpG methylation analysis using RRBS in obese men at three time points; pre-GPB, GBP-1w, and GBP-1y. See Tables S8, S9, and S10 for the full lists of genes. (A) Clinical characteristics at the three time points (arrows, GBP-1w versus pre-GBP or GBP-1y versus pre-GBP) (homeostatic model assessment of insulin resistance, HOMA-IR; high-density lipoprotein cholesterol, HDL; glucose at fasting, Glucose). See Table S7 for all clinical characteristics. Venn diagrams showing the amount of genes, with at least one differentially methylated CpG, that overlap when comparing changes between the three time points (B) and the amount of genes that overlap between Obese versus Lean and pre-GBP versus GBP-1y (C).

(D) Genomic views of differentially methylated genes implicated in the genetics of obesity and common to the Lean versus Obese and the pre-GBP versus GBP-1y. The presence of SNPs within the regions is listed in the side tables and the arrows visualize their exact location.

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